Genetic Control of Cadmium Tolerance in *Drosophila melanogaster*

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Flies from a transgenic line of *Drosophila melanogaster* with two copies of the metallothionein allele Mtn^3 were more tolerant to cadmium than strains with only one copy of the gene. However, flies with the Mtn^3 allele were as tolerant as flies with the Mtn^1 allele, despite the level of expression of Mtn^1 being three times higher than that of Mtn^3. We propose that the substitution of Lys-40 (in Mtn^3) for Glu-40 (in Mtn^1) accounts for a reduction in binding affinity of Mtn^1, which offsets the increased expression levels. *Key words*: cadmium tolerance, *Drosophila melanogaster*, gene duplication, life span, metallothionein, *Mtn* gene, viability. *Environ Health Perspect* 103:1116–1118 (1995)

One of the two metallothionein genes in *Drosophila melanogaster*, *Mtn*, occurs in two predominant alleles: Mtn^1, which was found at frequencies of 85% and 95% in American and European samples, respectively (*1*–3), and Mtn^3, which was the minority allele in the American and European samples, but was fixed in a sample from Congo (*2*). Structurally, Mtn^1 differs from Mtn^3 in three respects: 1) it has two base substitutions in the promoter region, 2) it lacks a 49-base pair segment in the 3' untranslated region, and 3) it has a base substitution in the C-terminal codon. Studies in sibling species suggest that Mtn^3 is the ancestral allele. RNA measurements indicate that Mtn^1 strains accumulate mRNA at a level approximately three times greater than do Mtn^3 strains, probably the consequence of one of the first two differences listed above (*2*).

Metallothionein seems to be a very monomorphic protein in most species. To our knowledge, *Drosophila* presents the only case in which genetic polymorphism of metallothionein has been studied; we have observed polymorphism both in the coding sequence and the presence of duplications (*2,4*). The occurrence of Lys-40 in Mtn^3 in place of the Glu-40 found in Mtn^1 may have a profound effect. This C-terminal residue is next to one of the Cys-*X*-Cys groups responsible for metal binding. Coordination of Cd^{2+} ions by the thiolate groups of four cysteine residues creates an excess negative charge that is thought to be neutralized by basic amino acids in the vicinity (*5*). Thus, the presence of Glu in that position may affect the binding capacity of the metallothionein produced by Mtn^1. Despite numerous attempts, it has not been possible to purify *Drosophila* metallothionein to test this hypothesis, so we resorted to the indirect approach of measuring tolerance to metal toxicity in flies of various genotypes.

Increased transcriptional activity in any of several duplications of the Mtn^1 allele is always accompanied by increased tolerance to cadmium and copper ions (*4*). The lower level of expression of Mtn^3, therefore, should lead to reduced metal tolerance, unless its potentially more efficient protein counters this effect. This argument assumes that the two alleles have comparable translational efficiency.

To discover whether altered levels of Mtn^3 product lead to correspondingly modified tolerance to toxic metals, we generated a transgenic line of *Drosophila melanogaster* with two copies of the Mtn^3 allele. As in the case of Mtn^1 natural duplications, flies with this synthetic duplication were more tolerant to cadmium than strains with only one copy of the gene. Comparison of the cadmium tolerance of Mtn^1 and Mtn^3 strains, however, showed that the two strains are quite similar; this suggests that the increased transcript level in Mtn^1 exactly compensates for a reduction in either its metal-binding efficiency or its stability, caused by the amino acid substitution.

Materials and Methods

Flies of a Canton S strain with allele Mtn^1 were crossed to a Samarkand strain (S500) with allele Mtn^3, and females of the progeny were back-crossed to Samarkand males. Females of this second generation were then individually mated to Samarkand males. After allowing these females to lay eggs for a few days, DNA was extracted from them and tested for presence of the Mtn^1 allele by Southern blotting. Vials with progeny from females that were heterozygous were kept, and vials in which the female proved to be homozygous for Mtn^3 were discarded. This procedure was repeated for 11 generations, at which time Mtn^1 homozygous stocks were established. Thus, stocks were created in which the majority of the genetic background was the same as that in the Samarkand Mtn^3 stock but which were homozygous for the Mtn^1 allele. Two of these lines, SCS1 and SCS2, were used for tests of metal tolerance.

Stocks with a duplication for the Mtn^3 allele were produced by P-element-mediated transformation with a vector carrying a copy of the Mtn^3 allele including 370 base pairs (bp) of the promoter region. We have shown that this segment of the promoter is sufficient for normal expression of the *Mtn* gene (*6*). Transformed flies were crossed to a strain carrying Mtn^3, and the third-chromosome marker, "red" (C301). The strain derived from this cross, C313, was homozygous for the Mtn^3 allele at two different sites. Level of expression was determined by northern analysis as previously done (*4*); we determined that the amount of RNA loaded in all lanes was comparable by visual inspection of ethidium-bromide-stained rRNA after electrophoresis.

Males, eclosed over a 24-hr period, were sorted in groups of 20 and kept on normal food for a 6-hr period. At this point they were transferred to treatment vials containing Instant Drosophila Medium (Carolina Biologicals, Burlington, North Carolina) supplemented with 0, 0.1, 0.5, or 1.0 mM $CdCl_2$. Surviving flies were counted every 24 hr and transferred to fresh vials with the corresponding medium every 3 days. We determined the half-life of different genotypes in the various treatments from survival curves. All genotypes and concentrations tested were tested simultaneously, with 4 vials of 20 flies for each genotype and each treatment. This unit experiment was repeated three times for each comparison.

Results and Discussion

Using P-element-mediated transformation, we produced flies carrying an extra copy of the Mtn^3 allele. Estimates of metallothionein RNA, by northern analysis, confirmed that these transformed flies (C313)

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We are grateful to Donna Maroni for helpful editorial comments. This research was supported with a grant from the National Institute of Environmental Health Sciences PHS ES02654.

Received 6 July 1995; accepted 11 August 1995.

accumulate higher levels of *Mtn* RNA than flies with a single copy of the gene (C301) (Fig. 1).

Flies with two copies of the Mtn^3 allele are more tolerant to cadmium than flies with a single copy, as is shown in Figure 2 and Table 1. Tolerance here is measured as the half-life of adult males in various media; Figure 2A shows a set of survival curves of the type used to obtain the half-life values reported in Figure 2B. These results indicate that the allele Mtn^3 is capable of participating in metal detoxification, and we propose that the failure to find duplications for this allele in natural populations is probably due to its infrequent occurrence in Europe. It is in Europe where duplications of Mtn^1 seem to have originated, in response to the agricultural use of copper salts for antimicrobial purposes (*2*).

In addition to studying flies with one and two copies of Mtn^3, we also determined the tolerance of a new allele, Mtn^{K45} (CK45), which has the same sequence and RNA level as Mtn^3 (data not shown) but which has an insertion of 14 bp (GTTACAATCGTTAC) in the 3' untranslated region (111 bp downstream of the termination codon). With respect to cadmium tolerance, Mtn^{K45} is indistinguishable from Mtn^3.

Table 2 and Figure 3 show that there is no significant difference between Mtn^1 (SCS1 and SCS2) and Mtn^3 (S500) flies with respect to their tolerance to various concentrations of cadmium. At the lowest cadmium concentration (0.1 mM), Mtn^1 appears to have a slight advantage, but analysis of variance indicates that overall the two alleles are not significantly different. Table 2 also shows that both Mtn^1 and Mtn^3 are more sensitive to cadmium than a duplication for Mtn^1 (statistically significant difference), as was expected based on our previous work (*4*). In Table 2, the half-life of flies was standardized as a percent of their half-life in control medium.

Despite a threefold difference in expression level of Mtn^1 and Mtn^3, the half-life in cadmium of flies carrying these alleles is not significantly different. Since the only known difference between the two alleles, other than in the level of expression, is in the substitution of Lys-40 (in Mtn^3) with Glu-40 (in Mtn^1), this result supports the hypothesis that the protein of Mtn^1 has reduced binding affinity or stability, which is compensated by its increased expression levels. It should be noted that metallothionein is probably quickly removed from the cytosol, either by aggregation or degra-

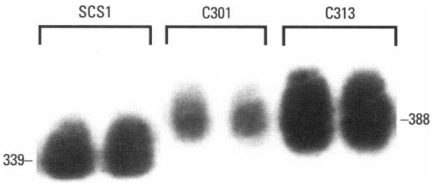

Figure 1. Autoradiograph of a northern blot using an *Mtn* cDNA probe and RNA extracted from flies induced with 1.0 mM $CdCl_2$ for 48 hr. Total RNA, 10 µg per lane, was used (*2*).

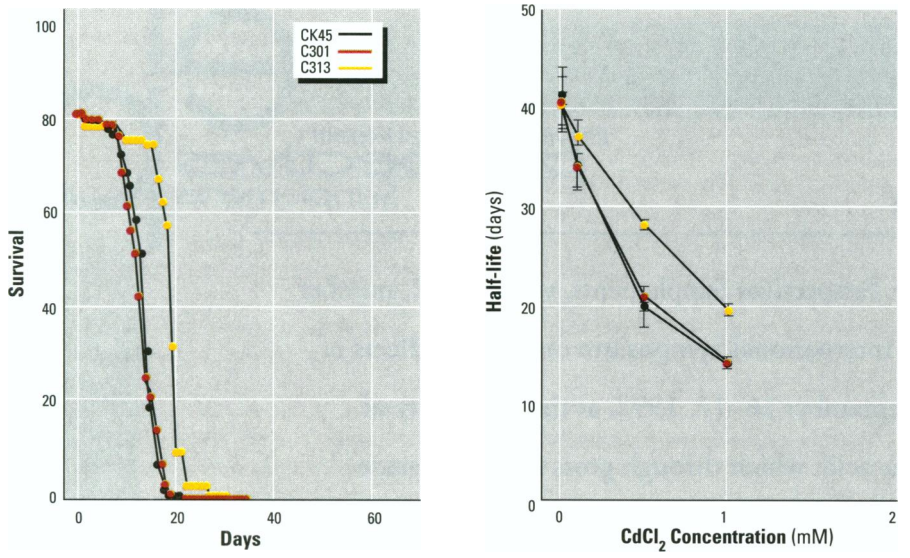

Figure 2. (A) Survival of adult males in medium supplemented with 1.0 mM $CdCl_2$. The data from four vials for each strain are combined here. Half-life estimates were obtained from plots such as this. For each strain and each $CdCl_2$ the experiment was repeated three times. (B) Half-life of adult males in medium supplemented with 0, 0.1, 0.5, or 1.0 mM $CdCl_2$. Vertical bars indicate standard errors.

Table 1. Half-life of adult male flies, in days, in medium with varying concentration of cadmium chloride[a]

	Mean (SE)			
Strain	Control	0.1 mM $CdCl_2$	0.5 mM $CdCl_2$	1 mM $CdCl_2$
C313 *[Dup(Mtn^3)]*	40 (3)	37 (2)	28 (0.5)	19 (1)
C301 *(Mtn^3)*	41 (3)	34 (2)	21 (1)	14 (0.5)
CK45 *(Mtn^{K45})*	41 (3)	34 (2)	20 (2)	14 (0.5)

[a] $N = 3$. Analysis of variance indicates that, with respect to cadmium tolerance, C313 *Dup(Mtn^3)* is significantly different from the other two strains ($p<0.01$), which are not significantly different from one another.

Table 2. Half-life of adult male flies, in days, in medium with varying concentrations of cadmium chloride, as percent of the half-life in control medium without cadmium[a]

	Mean (SE)			
Strain	Control	0.1 mM $CdCl_2$	0.5 mM $CdCl_2$	1 mM $CdCl_2$
DupH22 (Mtn^1)	100 (6)	112 (15)	92 (4)	59 (4)
SCS1 *(Mtn^1)*	100 (7)	82 (2)	55 (4)	34 (5)
SCS2 *(Mtn^1)*	100 (6)	78 (6)	48 (6)	33 (4)
S500 *(Mtn^3)*	100 (5)	98 (8)	52 (4)	37 (5)

[a] $N = 3$. Analysis of variance indicates that, with respect to cadmium tolerance, *DupH22 (Mtn^1)* is significantly different from the other three strains ($p<0.01$), which are not significantly different from one another.

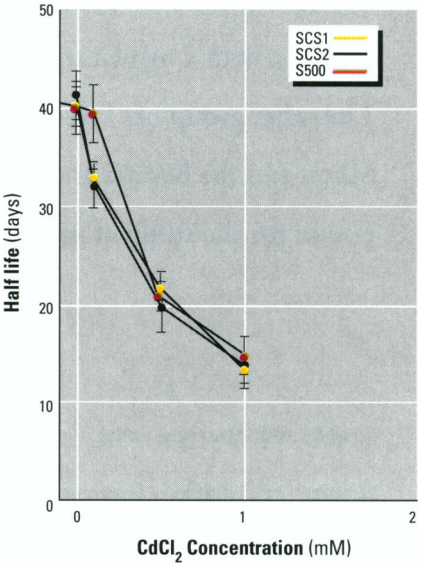

Figure 3. Half-life of adult males in medium supplemented with 0, 0.1, 0.5, or 1.0 mM $CdCl_2$. Vertical bars indicate standard errors.

dation, when not fully complexed with metal ions; we expect Mtn^1 and Mtn^3 flies to have comparable levels of soluble metallothionein, but larger amounts of Mtn^1 protein need to be synthesized to maintain that level.

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